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Production of a recombinant antimicrobial peptide in transgenic plants using a modified VMA intein expression system

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Abstract Tobacco plants were engineered to express SMAP-29, a mammalian antimicrobial peptide of innate immunity, as fusion protein with modified vacuolar membrane ATPase intein. The peptide was purified taking advantage of the intein-mediated self-cleaving mechanism. SMAP-29 was immunologically detected in the chromatographic eluate and appeared tightly bound to copurified plant proteins. Electrophoretic separation under disaggregating conditions indicated that the recombinant peptide was cleaved off by intein at the expected site and an overlay gel assay demonstrated that the peptide retained antimicrobial activity. These results indicate that a modified intein expression system can be used to produce pharmaceutical peptides in transgenic plants. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Antimicrobial peptide; Cathelicidin; Intein; Expression system; Transgenic plant; Biotherapeutic

1. Introduction

Inteins are protein splicing elements that can catalyse their self-excision from a precursor polypeptide triggering the fusion of the regions flanking the cleavage site (exteins) [1]. The intein-mediated self-cleaving mechanism has recently been exploited to purify recombinant proteins from bacterial cultures. Heterologous proteins can be synthesised in fusion to inteins that have been genetically engineered to promote a controllable fission of peptide bond at either terminus of intein itself. The release of the target protein can be obtained simply by adding nucleophilic agents such as 1,4-dithiothreitol (DTT), hydroxylamine or cysteine to generate the thioester intermediate required for the initiation of self-catalytic intein-mediated cleavage reaction. This strategy offers the main advantage that the protein of interest can be isolated from the bulk of cell extracts by a single-step purification [2,3].

The intein-based systems can be suitably designed for the production of many xenogenic proteins, such as hormones, enzymes, anti-viral and anti-infective peptides that need to be purified or downstream processed in order to be used as biopharmaceuticals [4,5]. The increasing demand for biopharmaceutical products has encouraged the search for new and commercially competitive systems for producing recombinant proteins. From this point of view, plants are an interesting source of heterologous proteins as they are able to replace almost any eukaryotic metabolic pathway and their application offers several advantages such as low production costs and lack of contamination from transmissible pathogen agents [6].

The aim of this work was to study the applicability of a self-cleaving intein expression system for producing xenogenic proteins in transgenic plants. The modified vacuolar membrane ATPase (VMA) intein expression/purification system has been applied to obtain recombinant SMAP-29, an α-helical cathelicidin peptide displaying a powerful antimicrobial activity [7–12], from transgenic tobacco plants. SMAP-29 was identified in sheep myeloid cells [7] and proved to be highly effective in vitro against a broad spectrum of bacteria and fungi [8]. It is noteworthy that SMAP-29 was also effective against antibiotic-resistant bacterial strains as well as *Pseudomonas aeruginosa*, a pathogen causing severe respiratory infections in cystic fibrosis patients [7–12]. Naturally occurring antimicrobial peptides have raised considerable interest for their potential as lead compounds for the development of new anti-infective agents [13,14] and they thus represent a good target for the application of intein-based systems in plants. The rationale of our work was to construct a transformation vector containing a transgene cassette made of SMAP-29 coding sequence [7] in conjunction with the modified VMA-1 intein cDNA derived from *Saccharomyces cerevisiae*, and the nucleotide tract encoding the chitin binding domain (CBD) of *Bacillus circulans* as affinity tag [2]. The *Agrobacterium*-mediated integration of the artificial gene into tobacco plant genome led to the expression of the fusion protein in transgenic plants. The β-conglycinin transit peptide (GenBank AJ276118) has been designed to address the fusion protein to endoplasmic reticulum and then to the apoplast compartment, in order to allow its recovery in the bulk of soluble proteins. The SMAP–intein–CBD polypeptide could be finally isolated by affinity chromatography using a chitin-derived matrix able to bind the CBD tag. The addition of nucleophilic compounds inducing the intein-mediated protein

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Abbreviations: AAU, acid-acetic-urea; BTP, bis-tris-propane; DTT, 1,4-dithiothreitol; ELISA, enzyme-linked immunosorbent assay; LB, Luria–Bertani; MW, molecular weight; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulphate; VMA, vacuolar membrane ATPase

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self-cleavage reaction led to the release of recombinant peptide.

2. Materials and methods

2.1. Plant expression vector, pBI-SMAP

Primer sequences used to build the vector (Fig. 1) and/or to perform molecular testing were:

1. 5′-CATTTGGAGAGACACCGGGG-3′
2. 5′-CCCGGGATGATGAGAGCGCGGTTC-3′
3. 5′-CTTAAGAGGGGACTTCGAAGACTG-3′
4. 5′-TCTAGACCCGGGTGCTTTGCCAAGGGTACC-3′
5. 5′-CGATCGGGGAAATTCGAGCTC-3′
6. 5′-GAGCTCTCATTGAAGCTGCCACAAGG-3′
7. 5′-CCCGGGCCCAGCTATTCTGATTATT-3′
8. 5′-CTTAAGGAGGTTGCAACGAG-3′
9. 5′-AATATACGCTATTGGAGCTGG-3′
10. 5′-ATGGCTCATTAAATCAGTTAT-3′

The intein–CBD sequence, isolated by PCR from pCYB 1 plasmid (NEB, Beverly, MA, USA) using primers 4 and 6, was cloned into pCR 2.1-TOPO vector (Invitrogen, Groningen, The Netherlands). Primer 4 introduced the *Xba*I and *Sma*I sites, whereas primer 6 the *Sac*I site. *Xba*I and *Sac*I were used for further cloning of intein–CBD (1572 bp) into pBI121 binary vector (Clontech, Palo Alto, CA, USA) after removal of the *uidA* gene. The intein–CBD encoding sequence was checked by the GeneBlast program to verify the compatibility between yeast and tobacco codon usage.

The β-conglycinin transit peptide sequence (192 bp) (GenBank AJ276118) was isolated by PCR from soybean genomic DNA using primers 2 and 8. Primer 2 introduced a *Sma*I site, while the sequence naturally terminated with the *Bfr*I site.

The SMAP-29 cDNA was isolated from sheep myeloid cell RNA by reverse transcription (RT)-PCR as [7] and cloned in BlueScript KS+ vector (Stratagene, San Diego, CA, USA). The whole template was amplified with primers 3 and 7 and cloned into pGEM-T (Promega, Madison, WI, USA). Primers added the *Bfr*I and *Sma*I sites, respectively. All the above-mentioned sequences were verified on both strands.

The 5′ terminus of SMAP-29 (99 bp) was fused to the 3′ terminus of the transit peptide sequence using the *Bfr*I site. This construct encoding β-conglycinin transit peptide and SMAP cDNA was then positioned at the 5′ end of intein–CBD in the pBI121 vector using the *Sma*I site. The PCR template spanning the whole transgene cassette and obtained using primers 1 and 5 is 1901 bp long.

The final pBI-SMAP vector was then used to transform tobacco (*Nicotiana tabacum* L., cv. Xanthi) leaf discs [15] with *Agrobacterium tumefaciens* EHA105 strain. Among putatively transformed (kanamycin-resistant) plants, 20, denoted T_1-T_{20}, were chosen for molecular testing.

2.2. PCR and Southern blotting of genomic DNA

PCR assays were performed with genomic DNA using primers 1 and 5. DNA size was evaluated by Molecular Weight Marker III (Boehringer-Mannheim, Germany). For Southern blotting, 10 μg of genomic DNA digested with *Hin*dIII (which cuts at the polycloning site and at the 467th base of intein sequence) was processed as in [16]. *SMAP–intein* cDNA probe was obtained by PCR amplification with primers 3 and 6.

2.3. RT-PCR and Northern blotting of total RNA

Total RNA, extracted from leaf samples by RNAgents Total RNA Isolation System (Promega), was retrotranscribed and amplified by Access RT-PCR System (Promega) using 2 or 3 as forward and 6 as reverse primers. RNA size was evaluated by Molecular Weight Marker III (Boehringer-Mannheim). For Northern blotting, 30 μg of total RNA separated under denaturing conditions was transferred onto a nylon membrane and hybridised with the above-mentioned *SMAP–intein* cDNA probe. After autoradiography cDNA probe was stripped off and RNA blots rehybridised with control ribosomal cDNA 550 bp probe; the latter was obtained by RT-PCR using primers 9 and 10.

2.4. Protein chromatography

Leaf tissue (5 g), powdered under liquid nitrogen, was incubated for 1 h at 4°C with 10 ml extraction buffer (50 mM bis-tris-propane (BTP), pH 9, 20 mM NaCl, 1 mM EDTA, 1% Triton X-100, 5% glycerol) containing Protease Inhibitor Cocktail (P9599 Sigma-Aldrich, St. Louis, MO, USA). The homogenate was centrifuged at $12\,000\times g$ for 30 min at 4°C, and the supernatant centrifuged again at $35\,000\times g$ for 1 h at 4°C. The clarified extract was subjected to a G-50 Sephadex column to separate intein fusion protein from the possibly in vivo cleaved peptide. The flow-through was concentrated and desalted using Centriplus YM-10 (Amicon, Millipore, Bedford, MA, USA). The extract was diluted 1:1 with column buffer (50 mM BTP, pH 9, 500 mM NaCl, 1 mM EDTA, 0.1% Triton X-100), loaded onto a 10 ml column of chitin resin (NEB) and washed with 10 volumes of column buffer. The intein-mediated self-cleavage reaction was induced by 24 h incubation at 4°C in cleavage buffer (50 mM BTP, pH 9, 20 mM NaCl, 1 mM EDTA) containing 50 mM DTT. The chromatographic eluate was collected and ultrafiltrated using YM-1 Amicon (Millipore) membrane. Samples were lyophilised and resuspended in 0.1% trifluoroacetic acid.

2.5. Protein electrophoresis and immunological assays

Protein samples were separated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) or acid-acetic-urea (AAU)–PAGE as in [17]. For fusion protein detection, 2 mg of crude extract proteins was mixed with 200 μl of chitin resin and incubated for about 4 h, at 4°C. The protein fraction bound to the chitin beads was recovered, washed with column buffer and stripped off with 200 μl of 6 M guanidinium containing 0.5% (w/v) *N*-lauryl sarcosine. After separation from the beads and acetone precipitation, proteins were dissolved in SDS–PAGE sample loading buffer and electrophoresed.

Western blotting and enzyme-linked immunosorbent assay (ELISA) were done following standard molecular techniques [16]. Briefly, for Western blotting, 200 ng of purified protein or synthetic peptide was electroblotted to Immobilon P^{SQ} membrane (Millipore). Blots were probed with 1:1000 dilution of polyclonal SMAP-29 antiserum, raised in rabbit, 1:10000 dilution of goat anti-rabbit immunoglobulins–peroxidase conjugate (Vector, Burlingame, CA, USA) and developed using the Supersignal PicoWest Chemoluminescent Substrate (Pierce, Rockford, IL, USA) detection system. For ELISA, protein samples were diluted in 0.1 M sodium bicarbonate buffer, pH 9.6, and seeded in a 96-well microtiter plate. After overnight incubation at 4°C, detection was made with 1:1000 dilution of rabbit polyclonal SMAP-29 antiserum, 1:5000 goat anti-rabbit biotinylated immunoglobulins (Vector). The reaction was enhanced by streptavidin–peroxidase conjugate (Sigma-Aldrich) and detection made using the Sigma-Fast *p*-nitrophenyl-L-phosphatase (Sigma-Aldrich) colorimetric system. Synthetic SMAP-29 peptide (RGLRRLGRKIAHGVKKYGPTVLRIIRIA), chemically obtained by a solid phase method [8], was used as positive control. The molecular size markers used were Sigma M 3546-Ultra Low Range (Sigma-Aldrich, St. Louis, MO, USA) and Bio-Rad 161-0304 Low Range (Hercules, CA, USA).

2.6. Gel overlay assay

The assay was carried out according to [18] with some modifications. A single clone of JM101 *lacZ*$^+$ *Escherichia coli* bacterial strain containing self-ligated pGEM-T vector (Promega) was grown overnight at 37°C in full strength Luria–Bertani (LB) medium. A mid-logarithmic phase subculture was obtained from an aliquot of stationary phase culture by a 2 h incubation at 37°C in fresh medium. Bacteria were collected by centrifugation, washed and resuspended in 10 mM NaPi buffer, pH 7.4. According to the relation $OD_{620\,nm}$ $0.2 = 5\times10^7$ colony-forming units (CFU)/ml, 4×10^6 CFU/ml was added to 1% (w/v) agarose/LB medium containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside and isopropyl-β-D-thiogalactopyranoside and poured into a Petri dish to form an underlayer gel, 1 mm deep.

AAU minigel and protein samples (500 ng of purified protein, 1 μg of synthetic peptide) were prepared and processed as described in [18]. After protein transfer, underlayer agarose gel was overlayed with a double-strength LB medium containing 1% (w/v) agarose and incubated for 24 h at 37°C until the zones of clearing were evident against the blue background. Bacterial growth inhibition areas were enhanced by Coomassie staining.

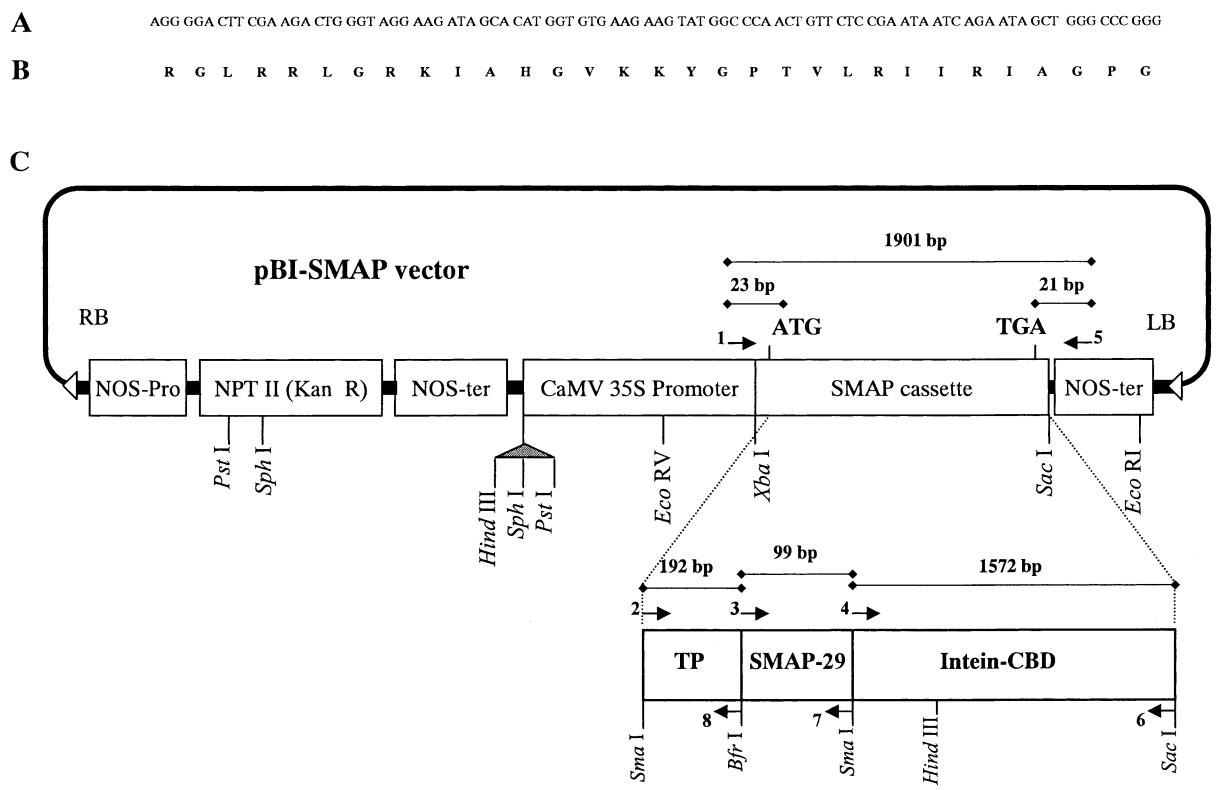

Fig. 1. Transgenic SMAP-29 nucleotide (A) and amino acid (B) sequence. C: pBI-SMAP transformation vector. The numbered arrows refer to the primers used for PCR and RT-PCR assays and the size of the amplification products (including the restriction sites added for cloning) is indicated (TP: β-conglycinin transit peptide; NOS-pro: nopaline synthase promoter; NPT II (Kan R): nopaline phosphotransferase II gene conferring kanamycin resistance; NOS-ter: nopaline synthase terminator; CaMV 35S: cauliflower mosaic virus 35S).

3. Results and discussion

3.1. Molecular analysis of transgenic tobacco plants

pBI-SMAP vector (Fig. 1) was constructed and used to achieve transgenic tobacco plants via *Agrobacterium* infection. The *SMAP–intein–CBD* cassette was cloned downstream of the constitutive enhanced CaMV 35S promoter and the β-conglycinin transit peptide encoding sequence. This latter should address the newly synthesised heterologous protein to the apoplast.

PCR analysis of genomic DNA confirmed the presence of the full-length transgene (1901 bp) in all the kanamycin-resistant plants, each deriving from an independent transformation event (Fig. 2A). Southern blotting revealed the insertion of one to four copies of the transgene in the tobacco genome (Fig. 2B). All plants produced self-pollinated seeds that generated kanamycin-resistant progenies. Transgenic DNA was detected by PCR in the genome of these plants (data not shown), confirming stable inheritance through meiosis.

Transgene expression was demonstrated in transgenic plant leaves by both RT-PCR and Northern blotting. In the first instance, amplification products corresponding to transgene coding sequence were obtained (1671 bp) (Fig. 3A). The lack of any amplicon in a PCR performed using total RNA, without RT step, ruled out genomic DNA contamination in the RNA preparations (data not shown). Northern blotting hybridisation revealed the presence of a messenger RNA band specifically recognised by *SMAP–intein–CBD* cDNA probe. This band was detectable only in RNA samples from trans-

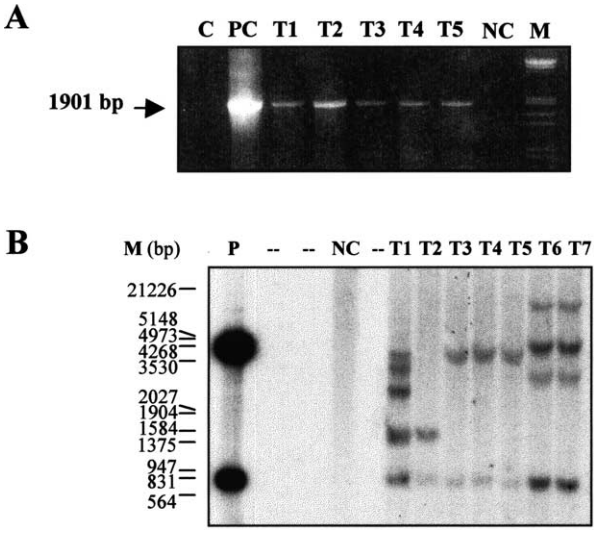

Fig. 2. Transgene integration in the genome of transformed tobacco. A: PCR detection of full-length transgene. B: Southern blotting of genomic DNA extracted from transgenic plants. M: molecular size marker, PC: pBI-SMAP (positive control), C: no DNA, NC: untransformed tobacco DNA (negative control), T_1–T_7: DNA of transformed tobacco plants, P: transgene SMAP cassette subcloned in pBI221 vector and digested with *Hin*dIII.

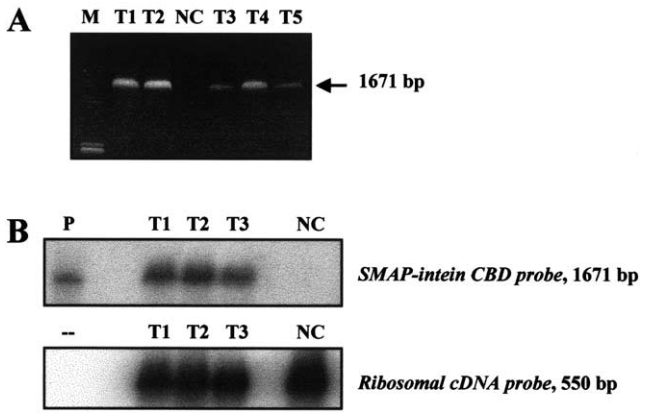

Fig. 3. Transgene expression in transformed tobacco leaves. A: RT-PCR detection of SMAP–intein–CBD mRNA using primers 3 and 6. B: Northern blotting of total RNA extracted from leaf tissue of transgenic plants M: molecular size marker, NC: untransformed tobacco RNA (negative control), T_1–T_5: RNA of transformed tobacco plants, P: SMAP–intein–CBD cDNA probe.

genic plants and was of a size compatible with transgene transcript (Fig. 3B).

3.2. Isolation, purification and analysis of the recombinant peptide

Transformed tobacco plants were then examined for the presence of the SMAP-29 intein–CBD polypeptide. The plants used for this analysis were chosen among the single-copy transgene lines with the highest transgene expression levels, as estimated by Northern blotting. The fusion protein was isolated incubating transgenic leaf homogenates with chitin beads. The chitin-bound polypeptide was stripped off and analysed by Western blotting. A rabbit polyclonal SMAP-29 antiserum specifically immunodecorated a 58 kDa band, corresponding to SMAP-29–intein–CBD fusion protein (Fig. 4).

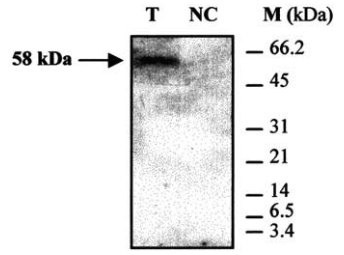

Fig. 4. Detection of SMAP–intein–CBD fusion protein by Western blotting with SMAP antiserum. T: transformed plant, NC: untransformed control plant (negative control), M: molecular size marker.

To purify SMAP-29 from the fusion protein, leaf crude extracts were loaded onto a purification column packed with chitin resin and the on-column intein-mediated self-cleavage mechanism was triggered by thiol addition. The CBD affinity tag was recognised by chitin molecule and allowed the isolation of SMAP-29 intein–CBD polypeptide, while thiol addition induced the intein-mediated protein splicing, thus causing release of recombinant peptide. Samples were recovered after elution and analysed by ELISA. SMAP antiserum revealed the presence of the peptide in the chromatographic eluate obtained from transgenic tobacco extracts, whereas no significant signal was found with the untransformed control processed under the same conditions (Fig. 5A). ELISA analysis did not reveal significant differences in the recovery of recombinant SMAP among eluate samples obtained after the chromatographic purification of extracts derived from different plants displaying comparable RNA levels (data not shown).

Purified samples were then separated by SDS–PAGE and subjected to Western blotting. SMAP antiserum detected a product with an apparent molecular weight (MW) (about 28 kDa) higher than synthetic SMAP-29 (3.2 kDa). The signal appeared to be specific since no band was detected in the

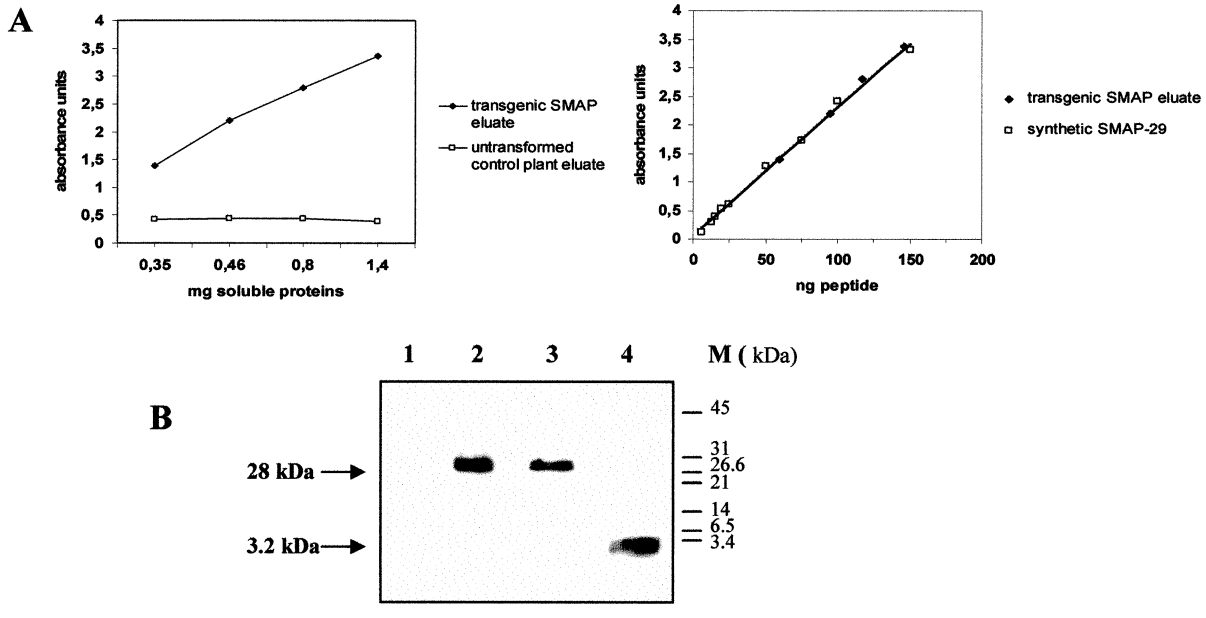

Fig. 5. ELISA and Western blotting analysis of the chromatographic eluate derived from purified tobacco leaf protein extract. A: Detection and quantification of transgenic SMAP-29 in the chromatographic eluate by ELISA assay. B: Western blotting of transgenic SMAP eluate treated with DTT. Lane 1: untransformed plant extract chromatography-purified eluate; lane 2: DTT-treated transgenic SMAP eluate; lane 3: transgenic SMAP eluate; lane 4: synthetic SMAP-29; M: molecular size marker.

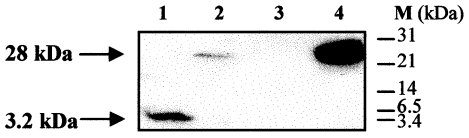

Fig. 6. Binding of synthetic SMAP-29 to plant proteins. SMAP-29 incubated in the presence of untransformed tobacco protein extract was separated onto 10–20% SDS–PAGE and analysed by Western blotting. Lane 1: 200 ng of SMAP-29; lane 2: untransformed plant crude extract incubated with 200 ng of SMAP-29; lane 3: untransformed plant crude extract; lane 4: SMAP eluate purified from transgenic plant extract; M: molecular size marker.

untransformed control eluate (Fig. 5B). The electrophoretic mobility of this product did not correspond to that of the fusion protein (58 kDa) nor to the unprocessed β-conglycinin transit peptide joined to SMAP-29. In addition, the occurrence of an aspecific cleavage was unlikely, since prolonged incubation of SMAP eluate in the presence of high DTT concentrations (100 mM, 48 h) did not produce additional bands (Fig. 5B, lane 2).

It has thus been investigated whether the presence of a 28 kDa band might be due to the interaction of recombinant SMAP-29 with copurified plant proteins. In fact, the tendency of cationic peptides to form higher MW complexes in association with serum proteins has previously been observed [19–22]. The ability of SMAP-29 to bind to plant proteins was first confirmed by incubating the synthetic peptide with the protein extract derived from the untransformed control. Western blotting demonstrated the added peptide bound to plant proteins, revealing a band with electrophoretic mobility similar to that of the complex in which the recombinant SMAP was recruited (Fig. 6). It is nonetheless worth noting that proteins contained in rabbit serum were also found to interact with SMAP-29 generating high MW complexes (data not shown).

Several attempts were then made to recover the peptide from the 28 kDa complex. These included the use of disaggregating agents such as guanidinium, detergents and high salt solutions (data not shown). However, a product with the same electrophoretic mobility as the synthetic peptide was recognised by SMAP antiserum only after treatment of the chromatographic sample with 20% DMSO (Fig. 7A). Similar results were obtained by resuspending SMAP eluate in AAU loading buffer in the presence of 10% (w/v) 3-cholamidopropyldimethylammonio-1-propane sulphonate. The separation of this sample under disaggregating conditions allowed the detection of a faint band with electrophoretic mobility similar to that of synthetic SMAP-29 (Fig. 7B).

To test the antimicrobial activity of the recombinant peptide, a gel overlay assay was done. This approach was used to demonstrate that SMAP-29 was functional and active after separation from the protein complex. The electrophoretic run under denaturing conditions separated the recombinant peptide disaggregated from the protein complex, while the transfer over a bacterial layer evidenced a bacterial growth inhibition area (Fig. 7C, lane 2) corresponding to the band of transgenic SMAP revealed by Western blotting (Fig. 7A) and similar to that obtained using the synthetic SMAP-29 molecule (Fig. 7C, lane 4). Conversely, the association of transgenic peptide with plant proteins resulted in a loss of bactericidal activity, as demonstrated by the lack of bacterial growth inhibition area in the overlay gel assay for the sample corresponding to SMAP eluate not disaggregated by DMSO treatment (Fig. 7C, lane 1). The ability of some plant proteins to strongly interact with SMAP-29 may thus be of some concern when purifying this peptide from transgenic plants. The

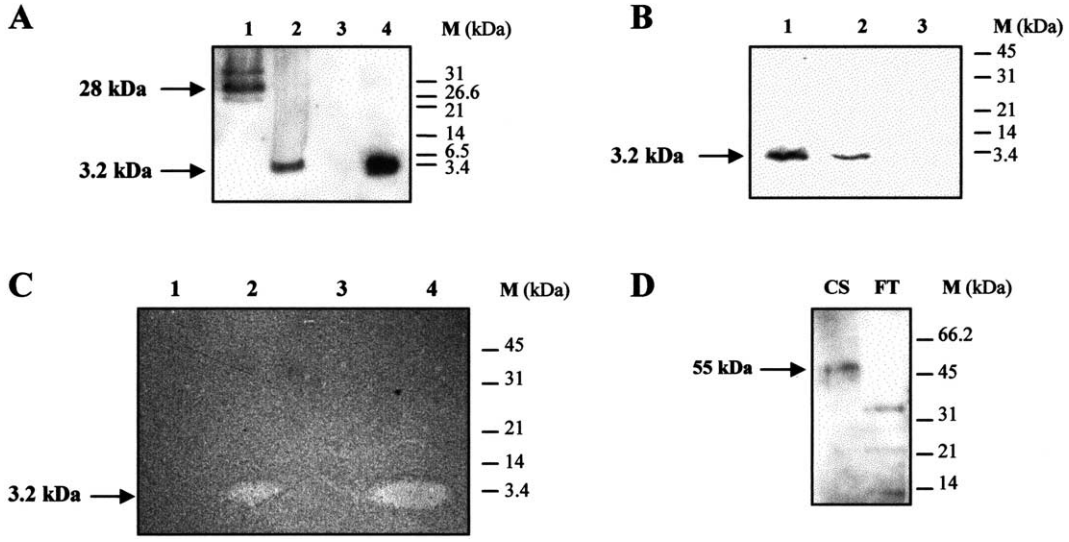

Fig. 7. Detection of disaggregated transgenic SMAP-29 peptide and evaluation of its antimicrobial activity. A: Western blotting of protein sample incubated in the presence of 20% DMSO, mixed to loading buffer and separated onto 10–20% SDS–PAGE. Lane 1: transgenic SMAP eluate; lane 2: DMSO-treated transgenic SMAP eluate; lane 3: DMSO-treated untransformed plant eluate; lane 4: synthetic SMAP-29; M: molecular size marker. Alternatively, (B) transgenic SMAP eluate was separated onto denaturing 18% AAU–PAGE. Lane 1: synthetic SMAP-29; lane 2: transgenic SMAP eluate; lane 3: untransformed plant eluate; M: molecular size marker. C: Antimicrobial activity of transgenic SMAP-29 detected by overlay gel assay. Lane 1: transgenic SMAP eluate; lane 2: DMSO-treated transgenic SMAP eluate; lane 3: DMSO-treated untransformed plant eluate; lane 4: synthetic SMAP-29; M: molecular size marker. D: Western blotting analysis of flow-through and column stripping fractions collected during transgenic plant extract chromatographic purification. FT: flow-through sample was the protein extract recovered after loading onto chitin-purification column; CS: column stripping sample after transgenic SMAP elution; M: molecular size marker.

purification problems that we faced may be related to structural features of SMAP-29, as suggested by the fact that treatment of the protein complex with a strong disaggregating agent resulted in disruption of the complex and release of a fully functional transgenic peptide of the same molecular size as the synthetic SMAP-29. Conversely, early steps in the intein-mediated expression mechanism were not problematic and resulted in the correct cleavage of the fusion protein, as demonstrated by the fact that the intein–CBD tail was recovered after column stripping and detected using CBD-specific antiserum (Fig. 7D, CS). Moreover, in the flow-through sample (Fig. 7D, FT), only aspecific bands with a MW lower than fusion protein size were detected. This indicates that the binding capacity of the chitin column allowed an almost complete recovery of the fusion protein from the total protein extracts.

3.3. Conclusions

This work provides the first evidence of the production of a recombinant protein in transgenic plants by exploiting the intein-mediated self-cleavage mechanism. This may represent a proof-of-concept study for a new biotechnological strategy for producing pharmaceutical polypeptides in plants. The results demonstrate that transgenic tobacco plants are able to produce a target peptide as a fusion protein with intein–CBD; this can be purified by affinity chromatography and the peptide released by inducing intein self-cleaving with nucleophilic agents. Although the system needs to be optimised according to the chemical features of the target product, these preliminary results clearly demonstrate that the self-cleaving intein expression is a promising strategy for producing peptides of pharmaceutical interest. Research is now in progress to increase the transgenic protein recovery rates and verify the applicability of the intein-based system to the synthesis of larger heterologous proteins in plants. In this case the efficiency of this purification strategy will be optimised in order to keep the correct protein folding and cleavage efficiency in the presence of multiple disulphide bonds.

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